

ORIGINAL ARTICLE

**REDUCED ANTIOXIDANT STATUS FOR PROLONGED
PERIOD DUE TO REPEATED STRESS EXPOSURE IN RAT**

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The objective of the study was to find out whether or not exposure to a stressor after an initial stressful experience augments stress response. Antioxidant status was determined by measuring changes in the activities of the hepatic free radical scavenging enzymes viz, superoxide dismutase (SOD), glutathione-S-transferase (GST), glucose-6-phosphate-dehydrogenase (G6PDH) and catalase (CAT) and levels of hepatic malondialdehyde (MDA) following exposure to 1 h restraint (RS) and after a gap of 4 h to forced swimming exercise (FS) in rats. The activities of hepatic CAT, SOD, G6PDH and GST were significantly reduced 2 h after RS compared to controls and 4 h after FS the activities of CAT and G6PDH remained at lower levels i.e. they were similar to those found after RS, whereas SOD and GST showed further significant decrease compared to those found after RS. On the other hand the MDA levels, indicative of lipid peroxidation were significantly increased after RS and showed further significant increase after FS. The results reveal that after initial stressful experience, the stress response is augmented due to exposure to another stressor whereas the system does not get habituated to stress exposure.

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Exposure to stressful situations is among the most common human experiences. It is reported that exposure to stress can stimulate many pathways leading to increased production of the oxygen free radicals (Adachi *et al.*, 1993; Liu, 1999; Irie *et al.*, 2000). These are formed in human body both in

physiological and pathological conditions in cytosol, mitochondria, lysosomes, peroxisomes and plasma membrane (Hemmani and Parihar, 1998). Psychological stress is associated with increased reactive oxygen species (ROS) production and oxidative damage and long term exposure to

psychological stressors may enhance the risk of many diseases like atherosclerosis, diabetes, rheumatoid arthritis and liver diseases (Kelly, 1999; Liu, 1999; Lu *et al.*, 2003). Stress plays a potential role in aggravating liver diseases viz., hepatic fibrosis and cirrhosis (Parolo and Rabino, 2001). Other types of stressors are also known to alter antioxidant status and cause tissue damage in experimental animals. For instance, in rats, immobilization for 6 h induced severe bleeding in the stomach and a significant increase in plasma levels of thiobarbituric acid (Liu *et al.*, 1994); 30 minutes cold immobilization increased thiobarbituric acid reactive substances (TBARS) and decreased concentration of sulfhydryl (SH) groups in the heart, stomach and brain without change in the liver (Kovacs *et al.*, 1996); immobilization for 8 h (Liu *et al.*, 1996) or 6 h (Oishi and Machida, 2002) caused a significant increase in lipid peroxidation (LPO), oxidative damage of nuclear DNA, decrease in number of neutrophils and activity of catalase (CAT) and superoxide dismutase (SOD); restraint for 6 h resulted in reduction in the level of reduced glutathione (GSH), oxygen radical absorbance and activity of SOD, glutathione-S-transferase activity (GST) (Zaidi *et al.*, 2006); 48 h cold stress (4°C) caused a significant increase in CAT, glutathione reductase (GR) and GSH and decreased SOD activity (Yuksel *et al.*, 2008), 30 minutes acute immobilization resulted in increased blood corticosterone and glucose levels, activities of MnSOD, CuZn SOD, CAT and glutathione peroxidase (GPx) whereas GR remained unchanged (Djorjevic *et al.*, 2010) and removal of whisker's lead to an increase in protein carbonyl formation and LPO in the brain, heart, liver, and spleen in mice which were maximized after 12 h (Wang *et al.*, 2007); In these studies alterations in the antioxidant status or oxidative damage have been studied after

exposure to a single stressor. It is possible that human beings or animals are exposed to several stressors within a day. Whether exposure to a stressor after an initial stressful experience further alters antioxidant status and thereby enhances oxidative damage or the system gets habituated to stress exposure remains to be understood. Hence the present study aims at investigating effects of restraint followed by forced swimming exercise applied after an interval of 4 h on the activities of CAT, SOD, GPx, GST and concentration of malondialdehyde (MDA) in rat.

MATERIALS AND METHODS

Adult male Wistar rats (25) weighing 180-200g were obtained from the inbred colony of the central animal facility of the University of Mysore and were maintained (3 rats/cage) under 12 h: 12 h light and dark cycle. The rats were divided into 3 groups viz., initial controls (n=5), treatment controls (n=10) and stress group (n=10) and were provided rat chow and tap water *ad libitum* during experimentation.

Five rats were killed prior to commencement of the experiment (initial control). The treatment controls were kept in home cage without any disturbance, whereas rats in stress group were restrained and later subjected to forced swimming exercise. The rats were restrained (RS) for 1 h by placing each rat in an open cylindrical glass restrainer measuring 6.7 cm in diameter and 22 cm in length and kept in a clean cage with bedding material. After a gap of 4 h each rat in stress group was forced to swim in a glass chromatography jar (18'' height & 8.75'' outer diameters) filled 2/3rd full of water (forced swimming exercise, FS) for 15 minutes at room temperature. Five rats in stress group and treatment controls were killed 2 h after RS and remaining rats were killed 4 h after FS.

At each autopsy the adrenal gland and the liver samples were collected and stored at -20°C until enzyme assays were conducted. The superoxide dismutase (SOD) (Marklund and Marklund, 1974), glutathione peroxidase (GPx) (Tappel, 1978), catalase (CAT) (Aebi, 1984), glutathione-S-transferase (GST) (Habig *et al.*, 1974), glucose-6-phosphate dehydrogenase (G6PDH) (Lee, 1982) activities and malondialdehyde (MDA) levels (Ohkawa *et al.*, 1978) were estimated in the liver sample. The adrenal gland homogenate was used for assay of 3β -hydroxy steroid dehydrogenase (3β -HSDH) activity (Shivanandappa and Venkatesh, 1997). All data were expressed as the mean \pm SE and one way analysis of variance (ANOVA) followed by Duncan's multiple range test were used to test the significant difference between mean values of different groups, fixing the minimum level of significance at $P < 0.05$.

RESULTS

There was a significant increase in the adrenal 3β -HSDH activity following RS compared to controls which showed further significant elevation after exposure to FS compared to RS (Fig1).

The liver CAT and G6PDH activities were significantly reduced following RS compared to initial controls as well as treatment controls and remained at this low level when measured 4 h after FS (Table 1).

The activities of the liver SOD and GST were significantly reduced following RS compared to initial controls and there was a further significant reduction after FS compared to RS group as well as respective controls (Table 1). There was no change in GPx activity either after RS or FS.

The MDA levels showed a significant increase following RS and further significant increase after FS compared to controls and RS group (Fig 2).

Table 1 Effect of restraint and forced swimming exercise on the activities of liver catalase (CAT), superoxide dismutase (SOD), glucose -6-phosphate dehydrogenase (G6PDH), glutathione- S transferase (GST) and glutathione peroxidase (GPX) in rat.

Group	CAT (nmol/mg/min)	SOD (U/mg protein)	G6PDH ($\mu\text{mol/mg/min}$)	GST ($\mu\text{mol/mg/min}$)	Gpx ($\mu\text{mol/mg/min}$)
Initial controls (Zero hour)	2.52 ± 0.86^a	19.77 ± 1.66^a	0.02 ± 0.01^a	30.9 ± 1.78^a	0.16 ± 0.005
2h after restraint (RS)	1.44 ± 0.87^b	13.07 ± 0.89^b	0.01 ± 0.01^b	24.69 ± 1.51^b	0.15 ± 0.12
Controls for RS group	2.66 ± 0.107^a	19.87 ± 2.02^a	0.02 ± 0.02^a	26.45 ± 1.52^{ab}	0.16 ± 0.006
4h after forced swimming (RS+FS)	1.26 ± 0.067^b	8.25 ± 2.06^c	0.01 ± 0.01^b	15.07 ± 1.38^c	0.13 ± 0.11
Controls for (RS+FS)	2.54 ± 0.128^a	20.28 ± 2.06^a	0.02 ± 0.01^a	28.05 ± 1.51^{ab}	0.16 ± 0.11
ANOVA F-Value (df=4,20)	47.686 $P < 0.001$	11.983 $P < 0.001$	10.286 $P < 0.001$	15.05 $P < 0.001$	NS

Note: All values are Mean \pm SE; Mean values with same superscript letters in a given column are not significantly different, whereas those with different superscript letters are significantly ($P < 0.05$) different as judged by Duncan's multiple test.

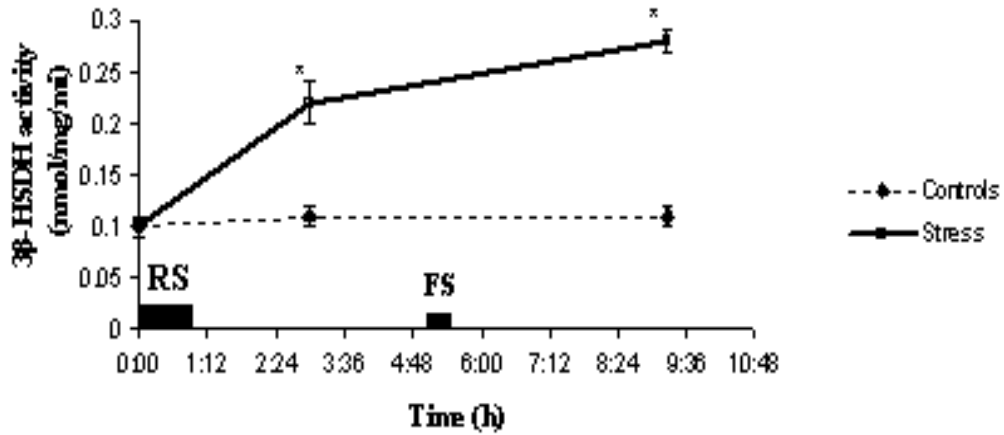


Figure 1 Changes in the adrenal 3β-HSDH activity following exposure to restraint (RS) and later to forced swimming exercise (FS) in rats. * Significant.

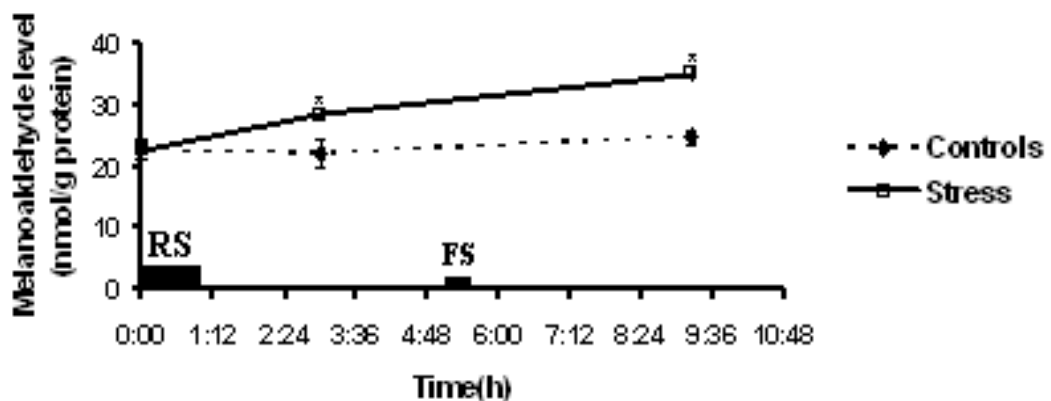


Figure 2 Changes in the malondialdehyde (MDA) levels following exposure to restraint (RS) and later to forced swimming exercise (FS) in rats. * Significant.

DISCUSSION

Increased adrenocortical activity is considered as an index of stress response in vertebrates as there is an unspecific activation of the hypothalamo-pituitary-adrenal axis (HPA axis) (Tsigos and Chrousos, 2002) due to stress. Hence, an increase in

the activity of 3β-HSDH, a key enzyme of adrenal steroidogenesis in the present study following RS as well as FS indicates that the animals were undergoing stressful experience.

Oxygen free radicals are extremely reactive and unstable and react with lipids, proteins, carbohydrates and nucleic acids in the body

(Sevanian and Hochstein, 1985) and generate a cascade producing lipid peroxidation (LPO), a major mechanism of cell membrane distraction and cell damage (Alession, 1993; Mapp *et al.*, 1995; Sato *et al.*, 1996; Ji, 1999). However biological systems have evolved endogenous defense mechanisms against free radical induced cell damage. Primary antioxidant enzymes viz. GPx, CAT and SOD directly eliminate active oxygen species (hydroxyl radical, superoxide radical, hydrogen peroxide) whereas glutathione reductase, G6PDH and cytosolic GST are secondary enzymes that detoxify ROS by decreasing peroxide levels or maintaining a steady supply of metabolic intermediates like glutathione and NADPH necessary for optimum functioning of primary antioxidant enzymes (Bandyopadhyay *et al.*, 1999). The generation of ROS is a primary event under a variety of stress conditions and the consequence of ROS formation depends on the intensity of the stress. Stressful conditions are known to interfere with antioxidant system by interfering with production or inactivation of antioxidant enzymes (Kono and Fridovich, 1983; Krotz *et al.*, 2002). Hence, changes in the activity of antioxidant enzymes are considered in the present study to assess the effect of repeated acute stress exposure. The SOD converts super oxide radicals into hydrogen peroxide. The ROS scavenging activity of SOD is effective only when its activity is followed by the actions of CAT and GPx, because hydrogen peroxide generated by SOD is further scavenged by CAT and GPx (Halliwell, 2001). Therefore, it is hypothesized that an imbalance in the SOD/CAT results in oxidative alterations. The reduction in the activities of SOD and CAT following RS and FS in the present study indicates decreased antioxidant status due to stress induced by restraint and forced

swimming. Further, decreased CAT activity indicates reduced hydrogen peroxide scavenging. In addition, there was also a decrease in potency of secondary antioxidant defense system in stressed rats as shown by decreased activities of G-6-PDH and GST. The fact that RS and FS caused oxidative stress is further supported by increase lipid peroxidation which is an indicator of oxidative damage to the cells. It is reported that lipid peroxidation is a major mechanism of the liver cell injury (Jaeschke *et al.*, 2002; Videla *et al.*, 2003) and is a consequence of imbalance between pro-oxidant and antioxidant system (Vijayavel *et al.*, 2005).

It is evident from earlier studies (references in introduction) that decreased antioxidant status was observed following one time exposure to an acute stressor. The present study, first time reveals that after a stressful experience, exposure to another stressor results in an enhanced stress response whereas the system does not get habituated to stress exposure.

The facts that restraint induced decreased activity of SOD and GST were further significantly reduced following FS and increase in lipid peroxidation following RS was further increased after FS support this view. It is to be noted that animals or humans face several stressors in a day and such experiences might result in increased liver cell damage, as evidenced by increased LPO in the present study following exposure to second stressor, although after a gap of 4 h. Further in earlier studies, activities of antioxidant enzymes were determined immediately after exposure to stressors. Hence, it is not possible to ascertain whether the stress induced changes are short lived or persist for a longer duration. This is an important fact to be considered because an increase in duration of prevalence of

high levels of ROS might lead to increased damage to tissues due to membrane lipid peroxidation. In the present study there was a few hours time interval between completion of stress exposure regime and collection of samples for antioxidant enzyme assays and LPO, i.e., sample was collected 2 h after RS and 4 h after FS and significantly lower antioxidant status and increased LPO were observed. Since, the adverse effects on antioxidant enzymes prevailed a few hours even after completion of stress regime our study shows that stress effects on antioxidant system are not short lived.

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